Journal of Proteomics 154 (2017) 128–133

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Proteomic analysis of the Rett syndrome experimental model *mecp2*^{Q63X} mutant zebrafish

Alessio Cortelazzo [a,b,c,*,1], Thomas Pietri [d,1], Claudio De Felice [e,1], Silvia Leoncini [a,f], Roberto Guerranti [b,c], Cinzia Signorini [f], Anna Maria Timperio [g], Lello Zolla [g], Lucia Ciccoli [f], Joussef Hayek [a]

[a] Child Neuropsychiatry Unit, University Hospital, Azienda Ospedaliera Universitaria Senese (AOUS), Siena, Italy
[b] Department of Medical Biotechnologies, University of Siena, Siena, Italy
[c] Clinical Pathology Laboratory Unit, University Hospital, AOUS, Siena, Italy
[d] Ecole Normale Supérieure, Institut de Biologie de l'ENS, Paris, France
[e] Neonatal Intensive Care Unit, University Hospital, AOUS, Siena, Italy
[f] Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy
[g] Department of Ecological and Biological Sciences, University of Tuscia, Viterbo, Italy

ARTICLE INFO

Article history:
Received 8 September 2016
Received in revised form 9 December 2016
Accepted 20 December 2016
Available online 03 January 2017

Keywords:
Energy metabolism
Muscle
Oxidative stress
Rett syndrome
Zebrafish

ABSTRACT

Rett syndrome (RTT) is a severe genetic disorder resulting from mutations in the X-linked *methyl-CpG-binding protein 2* (*MECP2*) gene. Recently, a zebrafish carrying a *mecp2*-null mutation has been developed with the resulting phenotypes exhibiting defective sensory and thigmotactic responses, and abnormal motor behavior reminiscent of the human disease. Here, we performed a proteomic analysis to examine protein expression changes in *mecp2*-null *vs.* wild-type larvae and adult zebrafish. We found a total of 20 proteins differentially expressed between wild-type and mutant zebrafish, suggesting skeletal and cardiac muscle functional defects, a stunted glycolysis and depleted energy availability. This molecular evidence is directly linked to the *mecp2*-null zebrafish observed phenotype. In addition, we identified changes in expression of proteins critical for a proper redox balance, suggesting an enhanced oxidative stress, a phenomenon also documented in human patients and RTT murine models. The molecular alterations observed in the *mecp2*-null zebrafish expand our knowledge on the molecular cascade of events that lead to the RTT phenotype.

Biological significance: We performed a proteomic study of a non-mammalian vertebrate model (zebrafish, *Danio rerio*) for Rett syndrome (RTT) at larval and adult stages of development. Our results reveal major protein expression changes pointing out to defects in energy metabolism, redox status imbalance, and muscle function, both skeletal and cardiac. Our molecular analysis grants the *mecp2*-null zebrafish as a valuable RTT model, triggering new research approaches for a better understanding of the RTT pathogenesis and phenotype expression. This non-mammalian vertebrate model of RTT strongly suggests a broad impact of Mecp2 dysfunction.

1. Introduction

Rett syndrome (RTT, MIM 312750), a progressive neurodevelopmental disorder with a frequency of approximately 1:10,000 live births, is a leading cause of severe intellectual disability in the female [1]. The typical disease shows a period of 6 to 18 months of an apparent normal neurodevelopment followed by regression and progressive loss of acquired cognitive, social and motor skills [2]. *De novo* mutations, altering the function of the X-linked methyl-CpG binding protein 2 (*MECP2*) gene, are the main cause of RTT [3]. Although the *MECP2* functions are yet to be fully clarified, the encoded protein can act as either a transcriptional repressor or activator by binding to methylated DNA [4], and is able to modulate alternative RNA splicing [5] and miRNA processing [6].

Several experimental mammalian models of RTT recapitulating several features of the disease have been developed [7]. Non-mammalian models of RTT have been recently described, including *Drosophila* and *Danio rerio* [8,9]. In particular, zebrafish has recently gained much attention as a vertebrate model for human neurodevelopmental and neurodegenerative diseases [10], showing a number of unique advantages that include its strong genetics (*i.e.*, amenable to molecular manipulations of its genome) [11,12], a large repertoire of well-studied behaviors [13], imaging capabilities in larvae and access to early nervous system

* Corresponding author at: Child Neuropsychiatry Unit, University Hospital, Azienda Ospedaliera Universitaria Senese (AOUS), Viale M. Bracci 16, 53100 Siena, Italy.
E-mail address: alessio.cortelazzo@biologo.onb.it (A. Cortelazzo).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jprot.2016.12.010
1874-3919/

development [14]. The *mecp2*-null zebrafish model mirrors the defective motor behavior and sensory response observed in RTT patients [9,15].

Most studies have focused on defects linked to the development and maintenance of brain networks [16]. However, the fact that *MECP2* is broadly expressed, from the early stages of development with expression levels increasing progressively and becoming particularly enriched in neural tissues [17], and the large set of symptoms described in human patients [18], argues for pleiotropic roles of *MECP2*.

Hence, by using a proteomic approach in whole tissue samples from larvae and adults, we demonstrate that *mecp2*-null zebrafish exhibits changes in the expression of proteins mainly linked to the balance of the redox status, energy metabolism and muscle function.

2. Materials and methods

2.1. Animals

Zebrafish larvae and adults were maintained at 28.5 °C on a 14–10 h on/off light cycles. Larvae were grown accordingly to Westerfield [19]. All experimental procedures were performed at room temperature (21–23 °C). To minimize the effect of the genetic background variability on our analysis, we used the progeny of wild-type and homozygote mutant issued from incrossed zebrafish heterozygotes for the *mecp2*^{Q63X} null mutation (C to T transition in position 184 of the zebrafish cDNA, leading to the generation of a stop codon). The C184T mutation was identified by PCR using DNA extracted from fin clip (the primers 5′-AAAGGAAAGGCATGATGTGG-3′ and 5′-GTATCGCCAACCTTTTGGAA-3′ flank the position of the mutation), followed by sequencing. The loss of *mecp2* has been previously confirmed by Western blot analysis [15]. Gene sequencing for mutant, heterozygotes and wild type animals was added as Supplementary material 1. The whole tissue samples of WT and *mecp2*^{Q63X} (referred as RTT further in the text), larvae and adults were used for proteomic analysis. All procedures were carried out in compliance with the guidelines of *Le Comité d'Éthique pour l'Expérimentation Animale Charles Darwin*.

2.2. Sample preparation

Adult fishes were homogenized (whole tissues) in 7 M urea, 2 M thiourea and 4% CHAPS, on ice. Four volumes of ice-cold buffer per weight of tissue for the homogenization procedure were added. Approximately 1/4 of complete protease inhibitor cocktail tablet (Roche, ID: 11836153001) per fish was added to the homogenates, subsequently incubated 2 h under agitation at 4 °C. The samples were then centrifuged at ~21,000 ×*g* for 15 min at 4 °C and soluble fractions reserved, snap-frozen and kept at −80 °C until use. Larvae were harvested at 8 dpf and snap-frozen and kept at −80 °C until use. To extract proteins, they were first subjected to 3 freeze-and-thaw cycles of 20 s each. Samples were then ground with a pestle in 1.5 mL tubes and vortexed for 30 s in a lysis buffer consisting of 9 parts 20 mM Tris-HCl, pH 8.5, 20 mM NaCl and 1 part protease inhibitor cocktail. The samples were placed on a shaking table for 20 min at room temperature before spinning down cellular debris at 16100 ×*g* for 10 min at 4 °C. The supernatant was transferred to a fresh tube and frozen in −80 °C until use.

2.3. Two dimension gel electrophoresis (2-DE)

2-DE was performed according to Görg et al. [20]. Briefly, samples (60 μg) were combined with solubilizing buffer containing 8 M urea, 2% w/v CHAPS, 0.3% DTT, 2% immobilized pH gradient IPG buffer (GE Healthcare), and were separated using 18 cm pH 3–10 non-linear IPG strips (GE Healthcare), in an Ettan IPGphor apparatus system (GE Healthcare). After focusing, strips were combined with equilibration buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% w/v SDS, 30% v/v glycerol, and 1% w/v DTT for 15 min, followed by equilibrating in equilibration buffer with 4% w/v iodoacetamide and a trace of bromophenol blue for 10 min. The second dimension was performed embedding IPG strips and a molecular weight standard at the top of a 1.5 mm thick vertical polyacrylamide gradient gel (8–16% T) using 0.5% w/v agarose, in an EttanDalt Six Electrophoresis system (GE Healthcare). Protein spots from the 2-DE gels were visualized by silver staining, optimizing the exposure time in order to avoid overexposure of some gels with respect to others. Each sample was carried out in triplicate under the same conditions.

2.4. LC–ESI–MS/MS (proteomic analysis)

After MS compatible silver staining [21], protein spots were carefully excised and subjected to in-gel trypsin digestion according to Shevchenko et al. [22]. The supernatant containing tryptic peptides was dried by vacuum centrifugation. Prior to MS analysis, the peptide mixtures were redissolved in 10 μL of 5% formic acid. Samples were analyzed using a split-free nano-flow LC system (EASY-nLC II, Proxeon, Odense, Denmark) coupled to a 3D-ion trap (model amaZon ETD, Bruker Daltonik, Germany) equipped with an online nano-ESI sprayer (the spray capillary was a fused silica capillary, 0.090 mm o.d., 0.020 mm i.d.). All experiments and the mass spectrometer acquisition parameters were according to Timperio et al. [23]. Acquired collision-induced dissociation spectra were processed in DataAnalysis 4.0, and deconvoluted spectra were further analyzed with BioTools 3.2 software and submitted to Mascot search program (in-house version 2.2, Matrix Science, London, UK). A list of identified proteins (Supplementary Table 1), and the inclusion criteria for protein identification and selection (*i.e.* peptide sequences and the relative score, peptide charge status) are reported (Supplementary Table 2). Regarding validation method for MS/MS searches having a small number of spectra but high MASCOT scores, manual interpretation of peptide fragmentation spectra was used to validate protein assignments.

2.5. Data analysis

Electrophoretic gels were digitized and analyzed by ImageMaster 2D Platinum v7.0 software (GE Healthcare). Spot volume was expressed as a ratio of the percentage volume (%V) detected from the entire gel to minimize differences between samples (normalization). Only spots appearing in all gels of the same group were matched with those of the reference gel. The background was subtracted from all gels using the average-on-boundary method. Results were expressed as mean ± standard deviation to the mean (mean ± SD), and analysis of protein variations was performed by multiple *t*-test, with a False Discovery Rate (*q*) of 0.05 (GraphPad Prism 6.01, GraphPad Software, Inc., CA, USA; MedCalc 12.1.4, MedCalc Software, Mariakerke, Belgium). A two-tailed *p*-value of <0.05 was considered as statistically significant.

3. Results

We found a total of 322 ± 10 spots in wild-type and RTT larvae, and 377 ± 17 spots in wild-type and RTT adults. A cluster of 32 spots, corresponding to 20 proteins, were differentially expressed in homozygote *mecp2*^{Q63X} mutant zebrafish (Table 1, Fig. 1 and Supplementary data). A detailed list of RTT/WT ratios is also reported (Supplementary material 2 and 3).

3.1. Protein expression changes in RTT zebrafish at the larval stage

Significant expression changes at the larval stage were present for 16 proteins, the overwhelming majority of them being decreased as compared to the wild-type counterpart. The two proteins prdx2 and dj1, known to play an important role in *anti*-oxidative stress (OS) processes [24,25], were overexpressed in the RTT zebrafish larvae, whereas apoa1b was underexpressed.